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Hydrocarbon and Fatty Acid Composition of Cheese As Affected by the Pasture Vegetation Type

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ABSTRACT: The determination of the geographical origin of dairy products is an ongoing issue. In this paper the effects of botanical diversity of two pastures on the hydrocarbon and fatty acid composition of cheese fat were studied, over 2 years of experimentation. Two areas in the Italian southwestern Alpine region, dominated by *Trifolium alpinum* (T) and *Festuca nigrescens* (F) vegetation, respectively, were chosen, and milk obtained from cows grazing on these pastures was used to produce a semi-hard traditional cheese. Cheese samples showed a significantly different composition of most linear hydrocarbons, odd-chain (C15, C17, and C17:1) and unsaturated (*trans*-11,*cis*-15-C18:2, C18:3, C20:4n-6, C20:4n-3, and 20:5n-3) fatty acids, according to pasture type. The ratio between C_{29} and C_{27} linear hydrocarbons, unlike the absolute content of the single molecules, showed a good discriminating ability between the two pastures and was little affected by the natural variability due to the climatic and environmental factors.

KEYWORDS: *hydrocarbons, fatty acids, cheese, pasture*

INTRODUCTION

Traceability is a key issue in particular for Protected Designation of Origin (PDO) productions. As for the dairy sector, PDO cheeses have specific characteristics and are defined according to their geographical area of production and to the cheesemaking procedures. Different approaches have been carried out to assess the origin authenticity of dairy products.[1−9] Attention has mainly been focused on the identification and quantification of specific compounds, and very often terpenes have attracted the main interest as tracers of the animal's pasture-feeding diet. Terpenes have been detected and identified in dairy products for decades, and many studies have shown that milk and cheese from different productions and seasons can be differentiated.[3,4,6−9] However, milk terpene content is highly influenced by vegetation, climate, and length of the grazing season. Its variability, which also derives from analytical difficulties, challenges the reliability of using terpene fingerprints for tracing the origin of dairy products.[7,9,10]

A different approach to the traceability issue takes into account the nonvolatile hydrocarbon compounds present in the neutral lipid fraction of fat. In a previous paper[11] the suitability of both isoprenoid hydrocarbons and esters of isoprenoid alcohols for the discrimination between lowland and highland dairy products was shown. In particular, 1-phytene, 2-phytene, and neophytadiene seemed to be promising markers of the origin of dairy products. Hydrocarbons are minor components of the unsaponifiable fraction of edible oils and fats. Most fat matrices contain small quantities of linear, branched, saturated, unsaturated, and terpenic hydrocarbons, and studies have been carried out to characterize the hydrocarbon fraction of some foods.[12−19]

Moreover, many studies have been carried out on the influence of the feeding system on the fatty acid (FA) composition of milk fat, particularly with regard to the effects of different diets on the content of the unsaturated long-chain fatty acids, such as linolenic and conjugated linoleic acid.[7,8,20−24] These acids are claimed to have positive effects on human health, and many studies have been carried out to naturally increase, through cow feeding, their content in milk. Fresh green forage, having higher levels of polyunsaturated fatty acids with respect to silage, allows the production of milk with a high content of polyunsaturated fatty acids.[20,22] The effect of *Trifolium alpinum* and *Festuca nigrescens* on the FA composition of milk and cheese has been recently tackled by Falchero et al.,[25] who detected a significantly higher content of odd-chain (C15, C17, and C17:1) FAs in dairy samples deriving from *T. alpinum*-dominated pasture and a significantly higher content of α-linolenic acid in those deriving from *F. nigrescens*-dominated pasture. That paper reported the results of the first year of experimentation of the research project "Pro-Alpe" aiming at the characterization and protection of typical dairy products through the detailed definition of the *terroir* of the Italian Alpine mountains.

Our research is included in the same project, but our first objective was to verify if cheese obtained from milk of cows grazing on two different types of vegetation, under the same grazing management and in the same geographical site, could be discriminated on the basis of the composition of their hydrocarbon fraction. To verify the influence of the natural sources of variability, such as environmental conditions (e.g., climate, contribution of the minor botanical species) or characteristics

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of the herd, the experiment was repeated for two consecutive years.

In addition, the FA composition of cheese produced in the two years was carried out to confirm the results obtained on the samples of the first year of experimentation.[25] For this purpose all of the cheese samples were analyzed by applying analytical procedures useful to detect and quantify branched and *trans* fatty acids, as well.

MATERIALS AND METHODS

Experimental Design. The study was conducted on a dairy farm in the Italian southwestern Alpine region (2230−2240 m asl) during the summer period of 2007 and 2008 (July 11−August 4, both years). The pasture vegetation was surveyed to map surfaces covered by two different vegetation types considered interesting for dairy production. Every year the selected areas were fenced to create four paddocks (two for adaptation and two for the experimental trial) for each treatment (Figure 1). In both years of experimentation the vegetation of paddock

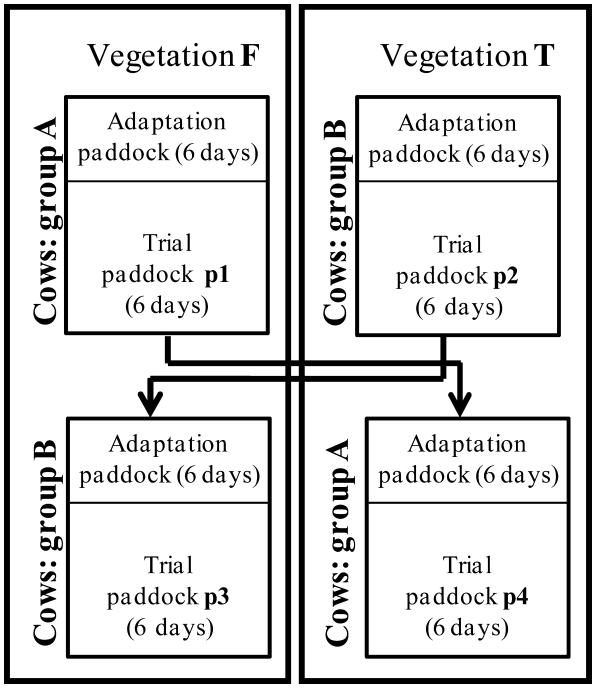

Figure 1. Experimental scheme: F, *Festuca nigrescens* pasture; T, *Trifolium alpinum* pasture.

F (p1 and p3) had a higher abundance of *F. nigrescens*, whereas in paddock T (p2 and p4) the vegetation was dominated by *T. alpinum*. Details of the composition of the two pastures were reported in previous papers.[25,26]

During the first year 18 multiparous cows in late lactation were involved in the experiment and divided into two groups, having the same number of heads of each breed. During the second year the herd was constituted of 12 multiparous cows in late lactation and divided into two groups, having each the same number of heads. Both years the two groups were balanced according to their mean daily production (16.4 ± 5.0 kg/cow·day). During the experimentation, the cows remained in the paddocks 24 h a day and received only mineral supplementation without any integration feeding. The experimental design, as reproduced in Figure 1, consisted of a 2 × 2 crossover design.[27] The herds underwent the two treatments. Both groups first spent 6 days in the adaptation paddocks, to enable the rumen to adapt completely to the vegetation type, and then were moved to the trial paddocks for the same treatment, grazing for another 6 days (period 1, group A = treatment F; group B = treatment T). The groups were then crossed over (period 2, group A = treatment T, group B = treatment F), and new paddocks were exploited in the same way for another 6 + 6 days.

Pasture Sampling. Every year the pasture sampling (T and F) was carried out 1 week before the entrance of the animals in the trial paddocks. During this week each paddock (p1−p4) was sampled twice, at the first and sixth days, and samples (100 g) were collected in triplicate.

Cheese Manufacture and Sampling. The cheesemaking followed the traditional steps and procedures adopted for the production of a semicooked and semihard traditional cheese, named *Nostrale d'Alpe*. The cows were milked twice a day at 7 a.m. and 6 p.m., in the paddocks, using a mobile milking machine. The bulk milk of the afternoon of each group was stored at 10 °C throughout the night and mixed with the bulk milk of the morning milking.

In particular, about 130 L of milk of each group was transferred into the vat and heated at 37 °C, and liquid calf rennet (25 mL/100 L) was added. Clotting time was about 40 min, and then the curd was first cut roughly. After 10 min, a second cut was carried out until the curd reached the size of a grain of rice. The curd stayed under whey for a few minutes and, after whey drainage, was placed into a mold and pressed. Cheese was then removed from the mold and manually salted on the surface.

After a ripening of 60 days at 10 °C and 85% relative humidity, cheeses were sampled, and a slice of about 250 g was taken from each whole cheese. The samples were stored at −20 °C until the analyses.

Twenty-four cheeses were produced each year: 12 from treatment F and 12 from treatment T. A total of 48 cheeses were obtained and analyzed throughout the two years of experimentation.

Reagents. High-purity standards of squalane (99%), squalene (≥98%), *n*-alkanes from C11 to C34 (99%), and methyl esters (purities ranging from 99.5 to 99.8%) and ethyl esters (purities ranging from 98 to 99%) of fatty acids, except for ethyl palmitate and ethyl oleate, were supplied from Sigma-Aldrich (Milan, Italy); phytane (99%) was purchased from Ultra Scientific (Bologna, Italy).

The *n*-hexane used in the whole extraction procedure of the hydrocarbon fraction was Suprasolv solvent (Merck, Darmstadt, Germany). All other reagents were of analytical grade and purchased from Sigma-Aldrich.

Chemical Composition. Dry matter, protein, fat, and ash contents of cheese samples were determined according to AOAC (2000) methods. Dry matter (DM) content of forages was determined by drying the samples in a forced draft oven at 60 °C to constant weight.

Cheese Fat Extraction. The method applied was developed at the laboratory to perform the fat extraction without using solvents.[11] Frozen cheese samples were thawed slowly at refrigerator temperature (4 °C) and finely grated. An amount of 20 g of grated cheese was weighed in a 60 mL screw-cap glass tube and warmed in a water bath at 60 °C for 20−30 min. Furthermore, the fat fraction was separated by centrifugation at 60 °C for 20 min at 1250 rpm (Gerber Instruments AG, Effretikon, Switzerland) and transferred into a glass vial.

Hydrocarbon Fraction Analysis. *Cheese Samples.* The hydrocarbon fraction was separated from the whole lipid matrix as described by Povolo et al.[11] and analyzed by GC-MS on a TraceGC coupled with a TraceMS Plus mass spectrometer (ThermoElectron Corp., Woburn, MA). A HP 5MS (Agilent Technologies, Palo Alto, CA) capillary column (30 m length, 0.32 mm i.d., 0.25 μm film thickness) was used. On-column injection (1 μL) was adopted, and helium was used as carrier gas at a flow rate of 1.5 mL min^{-1}. The oven temperature was held at 60 °C for 3 min, programmed to 280 °C at a rate of 10 °C min^{-1}, held at 280 °C for 1 min, programmed to 320 °C at a rate of 3 °C min^{-1}, and held at 320 °C for 20 min. The interface was held at 320 °C and the MS source at 250 °C. Acquisition was performed in EI mode (70 eV) by 1.0 scans s^{-1}, and the mass range used was 35−300 m/z^{-1} from 5 to 18 min, 35−470 m/z^{-1} from 18 to 28 min, and 35−600 m/z^{-1} from 28 to 60 min.

Identification of the compounds was made by using the NIST library (2001), the MS data of literature, the injection of authentic

Table 1. Mean Values and Standard Deviations of the Main Constituents (Percent Weight) of Cheese Samples Produced from Milk Deriving from the Two Vegetation Types (*F. nigrescens*; *T. alpinum*, T) in Two Different Years[a]

	vegetation type		year		
	F	T	1	2	
dry matter	62.39 ± 3.09	62.60 ± 3.28	64.81 ± 2.18	60.19 ± 2.12	**
fat	31.27 ± 2.11	32.19 ± 2.29	31.90 ± 2.35	31.57 ± 2.13	
protein	25.99 ± 1.95	25.38 ± 1.87	26.71 ± 1.42	24.65 ± 1.80	**
ash	3.93 ± 0.58	3.86 ± 0.47	4.17 ± 0.36	3.62 ± 0.52	**

[a]Significant difference: **, $p < 0.01$.

standards (when available), and the comparison of the retention indices with published data. Phytyl esters were previously identified.[11]

The quantification of the compounds was performed by relating the peak abundance to that of squalane (internal standard), and the amount was expressed as milligrams per kilogram of fat.

Pasture Samples. One hundred grams of fresh sample was added to 1.2 mg of internal standard (squalane) and extracted with three aliquots of 250 mL of dichloromethane. The solvent was evaporated under vacuum, and the extracted material was suspended in 10 mL of *n*-hexane before being loaded onto the same silica gel column applied for cheese samples. The column was eluted with 200 mL of *n*-hexane, and the eluate was collected, evaporated under vacuum to a small volume (1 mL), and analyzed by GC-MS with the same instrumentation and conditions described above for cheese samples.

Fatty Acid (FA) Composition. Fatty acids were determined as methyl esters, prepared by base-catalyzed methanolysis of glycerides using KOH in methanol.[28]

One microliter of the FAME solution was injected into a TraceGC (ThermoFisher, Rodano, Milan, Italy) gas chromatograph, equipped with a CP-Sil 88 (Varian, Santa Clara, CA) capillary column (100 m length, 0.25 mm i.d., 0.20 μm film thickness). A PTV injector in split mode (split ratio 1:100), at a constant temperature of 250 °C, was used, and hydrogen (0.5 mL min^{-1}) was adopted as carrier gas. The temperature program was as follows: 45 °C for 8 min, programmed to 173 °C at a rate of 12 °C min^{-1}, held at 173 °C for 47 min, programmed to 220 °C at a rate of 4 °C min^{-1}, and held at 220 °C for 20 min; a flame ionization detector (FID) was used, maintained at 250 °C.

The gas chromatographic response of short-chain fatty acids (C4−C10) was determined by preparing standard solutions of pure methyl esters (Sigma Chemical Co., St. Louis, MO) (when available) and injecting them by adopting the same instrumental conditions. Individual fatty acid methyl esters were identified by comparison to the standard mixture of Supelco 37 Component FAME Mix (Supelco, Bellefonte PA) and of CLA isomers (Sigma-Aldrich Co., St. Louis, MO). The identification of isomers of C18:1 was based on commercial standard mixtures (Supelco) and published isomeric profiles.[29]

Statistical Analysis. The evaluation of the effect of the different pasture and year was calculated by the analysis of variance (ANOVA). Multivariate analysis (principal component analysis (PCA)) was also applied to explore the data according to pasture type. Both statistical procedures were performed by using the XLSTAT 7.5 package (Addinsoft, France).

RESULTS AND DISCUSSION

The results for the main constituents of cheese samples are reported in Table 1. Differences statistically significant were detected for dry matter, protein, and ash contents, but only according to the year of production. The composition varied within the range usually observed for this type of cheese, which is produced in a artisanal way by a local cheesemaker, using a small amount of bulk milk.

Hydrocarbon Composition. Figure 2 shows the GC-MS chromatogram of the nonvolatile hydrocarbon fraction of a T (*T. alpinum*) cheese sample separated from the whole lipid matrix by liquid column chromatography. Among all of the compounds detected, linear and isoprenoid hydrocarbons and phytyl esters were taken into account, because in a preliminary work they had shown interesting correlations with the feeding.[11]

Isoprenoid hydrocarbons are plant secondary metabolites, biosynthetically formed by condensation of isoprene units. Squalene, as well as phytol derivatives, such as 1-phytene, 2-phytene, phytane, and neophytadiene, are the main components of this class of compounds in milk fat.[17,18] Phytenes are metabolically derived from phytol, present in ingested pastures as a part of the chlorophyll molecule, by the action of the microorganisms in rumen liquor. Phytol can be either dehydrated to neophytadiene, which is transformed to 1-phytene by hydrogenation, or directly hydrogenated to dihydrophytol, which can also originate 1-phytene via dehydratation.[30] Moreover, Urbach et al.[18] hypothesized that 1-phytene could be produced from neophytadiene present in pasture grass, further hydrogenated in the rumen, and then transferred into milk.

Linear-chain saturated hydrocarbons are plant secondary metabolites and reported to be important constituents of cuticular waxes. They are present as mixtures with chain lengths ranging from 21 to 37 carbon atoms, and they are mostly represented by odd-numbered homologues. In several pasture species the predominant alkanes were reported to be nonacosane (C_{29}), hentriacontane (C_{31}), and tritriacontane (C_{33}).[31] *n*-Alkanes, as well as all other long-chain aliphatic compounds present in plant cuticular waxes, originate from a series of metabolic steps belonging to the acetyl coenzyme A biosynthetic pathway. Once very long chain fatty acids are formed, they can undergo further reductive reactions, transforming them into sets of different compounds such as aldehydes, alcohols, and finally *n*-alkanes after the loss of one carbon atom.[32] The presence of these compounds in plants, as well as their function and regulation, seems to be related to protection mechanisms from environmental factors, such as prevention of water loss or of thermal or UV-related stress; perhaps it is significant that they were detected at relevant concentrations in plants growing at high altitude,[33] where environmental conditions are rather harsh.

The occurrence of linear-chain alkanes in bovine and swine tissues has been associated with the vegetables consumed by animals as a part of their diet.[13−15,34,35] The *n*-alkanes, being inert throughout the digestive tract, are commonly used as markers for the estimation of intake, digestibility, and diet composition in grazing animals.[36,37]

As for the fatty acid phytyl esters, as reported in our previous paper,[11] two reasonable hypotheses can be formulated: direct transfer from grass, because they had been detected in vegetable matrices, and/or endogenous (rumen or mammary cells) esterification product between fatty acids and free (*E*)-phytol, which is the main isomer naturally occurring in the chlorophyll molecule.

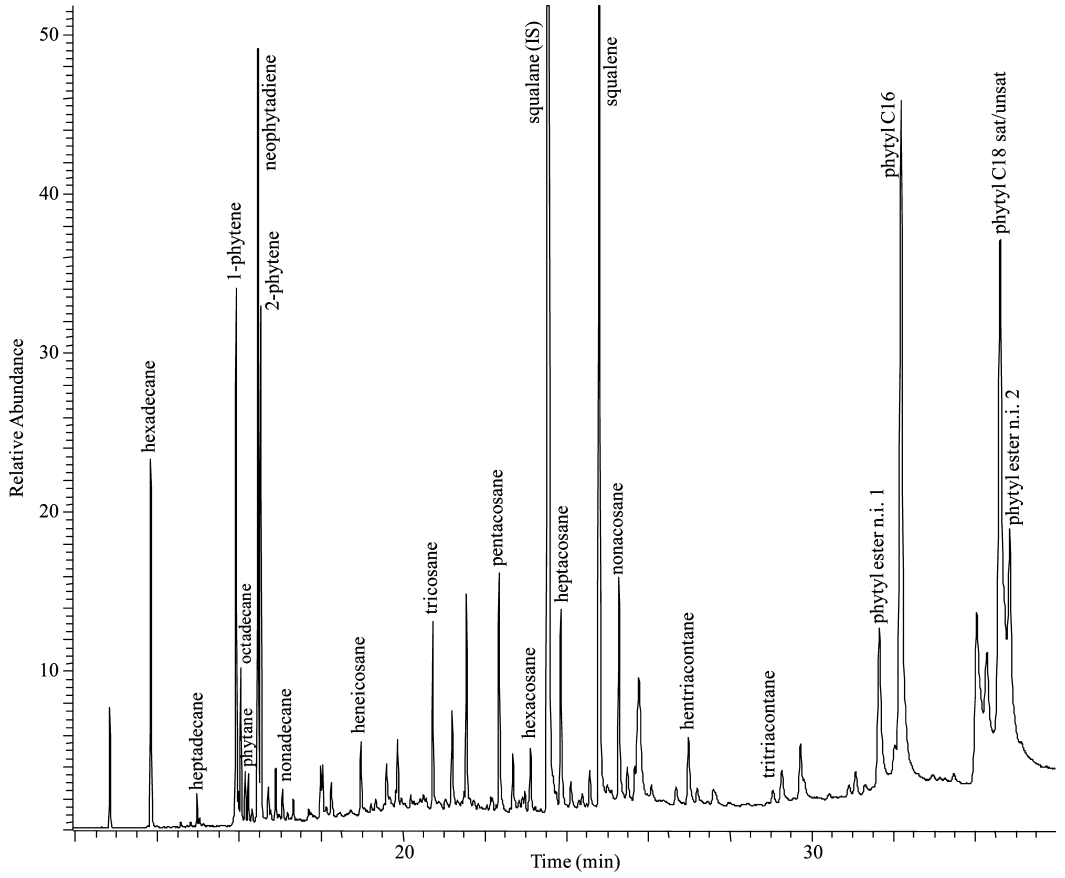

Figure 2. GC-MS profile of the nonvolatile hydrocarbon fraction of a *T. alpinum* cheese sample.

Before evaluation of the effect of the pastures, the total content of phytenes was calculated (1-phytene + 2-phytene + neophytadiene), to confirm the effectiveness of this index, developed by Povolo et al.,[11] for the distinction of dairy products deriving from milk of cows grazing on mountain pasture from those deriving from cows fed under intensive breeding systems in plain regions. The range of the values obtained on all the samples, independent of the type of pasture, varied between 20 and 80 mg kg^{-1} of fat and showed to be always higher than the values previously obtained[11] for dairy samples originating from plain regions (2.5−11.1 mg kg^{-1} of fat), thus confirming the discriminating power of this index.

As expected, due to the lack of significant differences for 1-phytene and 2-phytene between the F and T samples, the total content of phytenes did not show any discriminating ability with respect to the samples deriving from the two different pastures (Table 2).

Together with phytenes, most of the molecules showed significant differences according to the year of experimentation; this behavior can be explained by the different climatic conditions occurring during the two years, which are responsible for the different growths of the grass and consequently for the biosyntheses of the secondary metabolites. Other compounds were statistically different according to vegetation type, but they also showed a statistically significant difference on the basis of the year. Only a few compounds, belonging to the class of *n*-alkanes, seemed less influenced by the year. In particular, hexadecane (C_{16}), nonacosane (C_{29}), and hentriacontane (C_{31}) showed significant differences only with respect to the type of pastoral vegetation, even though it may not be excluded that their low concentration could have masked the effect of the year.

With the aim of finding one or more parameters able to discriminate the different pastures and not, or less, influenced by the different years of sampling, some ratios between the compounds were calculated and tested. The ratio C_{29}/C_{27} provided the most effective discrimination between the two groups of cheese. Cheese samples deriving from vegetation T showed a higher content of nonacosane than of heptacosane (C_{29}/C_{27} = 1.8 ± 0.5), whereas lower values of this ratio were observed for the samples belonging to the group of vegetation F (1.0 ± 0.2). To better display the differences obtained for the two categories of cheeses, the values of C_{29}/C_{27}, obtained for each sample, were subtracted from the overall mean of the values of the ratio (1.4), and results are drawn in a bar chart (Figure 3). With the exception of six samples, all of the samples deriving from pasture F showed positive values, whereas those deriving from pasture T showed negative ones. The real influence of the pasture on the hydrocarbon composition of cheese was then verified by analyzing the hydrocarbon fraction of T and F pasture samples.

The main components detected in the fresh pastures included linear odd-chain alkanes together with two isoprenoid compounds, named neophytadiene and squalene (Table 3). All of the compounds, except for neophytadiene, squalene, and tritriacontane (C_{33}), were influenced by the year, and three constituents (C_{25}, C_{27}, and C_{33}) were highly affected ($p < 0.01$) by the vegetation type. By comparison of the profile of odd-chain alkanes of pasture with that of cheese, important differences were observed (Tables 2 and 3). Independent of

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Table 2. Mean Values (Milligrams per Kilogram of Fat) and Standard Deviations of the Main Nonvolatile Hydrocarbons Detected in Cheese Samples Produced from Milk Deriving from the Two Vegetation Types (*F. nigrescens*, F; *T. alpinum*, T) in Two Different Years[a]

		vegetation type			year		
compound	identification method[b]	F	T		1	2	
hexadecane (C_{16})	MSa	0.6 ± 0.46	0.4 ± 0.20	*	0.4 ± 0.22	0.6 ± 0.47	
heptadecane (C_{17})	MSa	1.1 ± 0.60	1.1 ± 0.52		1.4 ± 0.55	0.7 ± 0.18	**
1-phytene	MSr/PI	21.2 ± 10.71	22.7 ± 12.66		31.7 ± 7.86	12.2 ± 3.90	**
octadecane (C_{18})	MSa	4.1 ± 1.64	3.5 ± 1.59	*	5.1 ± 1.17	2.5 ± 0.60	**
phytane	MSa	1.5 ± 0.84	1.4 ± 0.56		2 ± 0.59	0.9 ± 0.20	**
neophytadiene	MSr/PI	8.2 ± 2.48	8.1 ± 1.84		7.2 ± 1.69	9.1 ± 2.22	**
2-phytene	MSr/PI	15.6 ± 6.74	16.1 ± 5.61		21.1 ± 4.20	10.6 ± 1.56	**
nonadecane (C_{19})	MSa	0.6 ± 0.47	0.5 ± 0.28		0.7 ± 0.43	0.4 ± 0.30	*
heneicosane (C_{21})	MSa	2.3 ± 2.15	3.2 ± 3.82		4.2 ± 3.90	1.4 ± 0.55	**
tricosane (C_{23})	MSa	7.4 ± 5.24	4.9 ± 2.79	**	9.3 ± 4.17	3.0 ± 0.80	**
pentacosane (C_{25})	MSa	8.3 ± 3.71	12.5 ± 8.70	**	16.2 ± 5.32	4.6 ± 1.01	**
hexacosane (C_{26})	MSa	1.5 ± 1.27	11.3 ± 9.58	**	10.6 ± 10.28	2.2 ± 1.11	**
heptacosane (C_{27})	MSa	7.3 ± 2.74	6.0 ± 1.82	*	7.4 ± 2.89	5.9 ± 1.52	*
squalene	MSa	57.7 ± 37.30	62.7 ± 34.35		92.5 ± 19.13	27.9 ± 6.70	**
nonacosane (C_{29})	MSa	7.5 ± 3.65	10.2 ± 2.69	**	9.3 ± 3.82	8.4 ± 3.05	
hentriacontane (C_{31})	MSa	3.0 ± 1.05	3.5 ± 1.03	*	3.1 ± 1.10	3.4 ± 1.03	
tritriacontane (C_{33})	MSa	1.0 ± 0.33	0.9 ± 0.33		0.9 ± 0.31	1.0 ± 0.35	
phytyl ester n.i. 1[c]		9.1 ± 2.90	8.4 ± 2.69		8.7 ± 2.36	8.7 ± 3.21	
phytyl C16	MSa	41.2 ± 14.45	27.6 ± 8.61	**	41.3 ± 15.68	27.4 ± 5.77	**
phytyl C18 sat/unsat[d]	MSa	24.4 ± 7.67	18.0 ± 5.89	**	25.1 ± 8.20	17.3 ± 4.11	**
phytyl ester n.i. 2[c]		6.5 ± 2.55	4.6 ± 2.29	**	6.8 ± 2.95	4.2 ± 1.06	**
Σ phytenes[e]		45.0 ± 16.54	46.9 ± 17.68		60.0 ± 12.02	31.85 ± 5.51	**
C_{29}/C_{27}		1.0 ± 0.22	1.8 ± 0.50	**	1.4 ± 0.70	1.4 ± 0.38	

[a]Significant difference: *, $p < 0.05$; **, $p < 0.01$. [b]Confirmation of the identification: MSa, mass spectra of authentic compounds (authentic compounds had the same retention indices as the molecules detected in the samples); MSr, comparison with mass spectra reported in the literature;[18] PI, published indices, comparison of KI calculated with published indexes.[47] [c]See Figure 1. [d]Saturated/unsaturated. [e]Sum of 1-phytene + 2-phytene + neophytadiene.

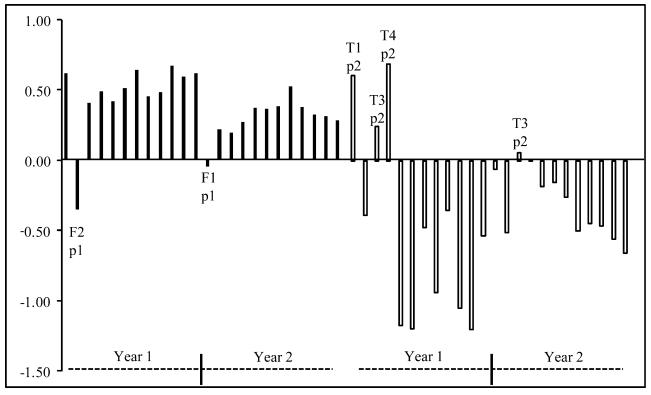

Figure 3. Ratio between the amount of C_{29} and C_{27} in cheese samples, expressed as 1.4-C_{29}/C_{27}: (black bars) *F. nigrescens* type (F); (white bars) *T. alpinum* type (T). p1−p4 identify the experimental paddocks indicated in Figure 1.

the type of pasture, C_{29}, C_{31}, and C_{33} were the most abundant in pasture samples, whereas C_{25}, C_{27}, and C_{29} were the most concentrated in cheese samples. A reasonable explanation of this behavior can be derived from the results of the studies aiming to evaluate the composition, intake, and digestibility of the diet of herbivores.[31,37,38] These studies are based on the determination of the concentrations of *n*-alkanes in feces, because the wax components of the different plant species have a different *n*-alkane quali-quantitative composition. An assumption inherent in this procedure is that *n*-alkanes are fully recovered in feces. However, the studies in this field clearly showed that fecal recovery was incomplete and progressively declined as carbon-chain length decreased. Dove and Mayes[39] established that this incomplete recovery was due to adsorption from the small intestine. The lowest proportion of C_{31} and C_{33} on the profile of *n*-alkanes in cheese samples, in comparison to the highest values of these molecules in the two pastures, demonstrated, according to the literature, that the adsorption of long-chain hydrocarbons (C_{31} and C_{33}) in the small intestine, and its corresponding transfer to milk, was very low, and it increased when the number of carbon atoms decreased.

The ratio C_{29}/C_{27} was then calculated on F and T pasture samples and provided a significant difference ($p < 0.01$) only with respect to the pasture type. The results were processed like those of cheese samples; that is, they were subtracted from the overall mean value (3.6) and drawn in a bar chart (Figure 4). Both F and T pastures showed the same positive and negative behaviors as F and T cheese samples with three exceptions. The detailed observation of the samples that in both matrices provided values in contrast with their own category, or very close to zero, allowed the detection of interesting correspondences. Cheese sample F1p1 (second year) derived from the first collection of milk of cows grazing pasture F in paddock 1, and pasture sample F2p1was collected in the same paddock 2 days before the entrance of the animals. Cheese samples T1p2, T3p2, and T4p2 (first year) derived from milk of cows grazing pasture T in paddock 2 that gave an outlier result at the first sampling (T1p2) and a value close to zero at the second sampling (T2p2). The same correspondence was observed for

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Table 3. Mean Values (Milligrams per Kilogram DM) and Standard Deviations of the Main Nonvolatile Hydrocarbons Detected in the Two Vegetation Types (*F. nigrescens*, F; *T. alpinum*, T) in Two Different Years[a]

		vegetation type			year		
compound	identification method[b]	F	T		1	2	
neophytadiene	MSr/PI	11.7 ± 6.45	12.1 ± 1.4		14.1 ± 6.51	9.7 ± 4.12	
heneicosane (C_{21})	MSa	3.0 ± 2.50	1.4 ± 1.25	*	3.5 ± 2.20	0.8 ± 0.39	**
tricosane (C_{23})	MSa	12.8 ± 8.85	8.6 ± 5.51		16.5 ± 5.94	4.9 ± 2.39	**
pentacosane (C_{25})	MSa	27.2 ± 17.48	13.6 ± 8.29	**	31.0 ± 14.26	9.8 ± 4.34	**
heptacosane (C_{27})	MSa	36.9 ± 20.43	23.9 ± 15.86	**	46.5 ± 12.22	14.4 ± 5.44	**
squalene	MSa	18.6 ± 10.35	10.2 ± 3.44	*	11.1 ± 8.11	17.7 ± 8.24	
nonacosane (C_{29})	MSa	90.5 ± 43.23	96.1 ± 49.64		132.8 ± 23.33	53.8 ± 15.30	**
hentriacontane (C_{31})	MSa	165.1 ± 96.99	94.1 ± 49.29	*	181.8 ± 83.98	77.4 ± 37.85	**
tritriacontane (C_{33})	MSa	196.6 ± 126.93	29.4 ± 19.23	**	143.7 ± 147.17	82.3 ± 93.02	
C_{29}/C_{27}		2.7 ± 0.81	4.5 ± 1.21	**	3.1 ± 1.12	4.1 ± 1.48	

[a]Significant difference: *, $p < 0.05$; **, $p < 0.01$. [b]Confirmation of the identification: MSa, mass spectra of authentic compounds (authentic compounds had the same retention indices as the molecules detected in the samples); MSr, comparison with mass spectra reported in the literature;[18] PI, published indices, comparison of KI calculated with published indexes.[47]

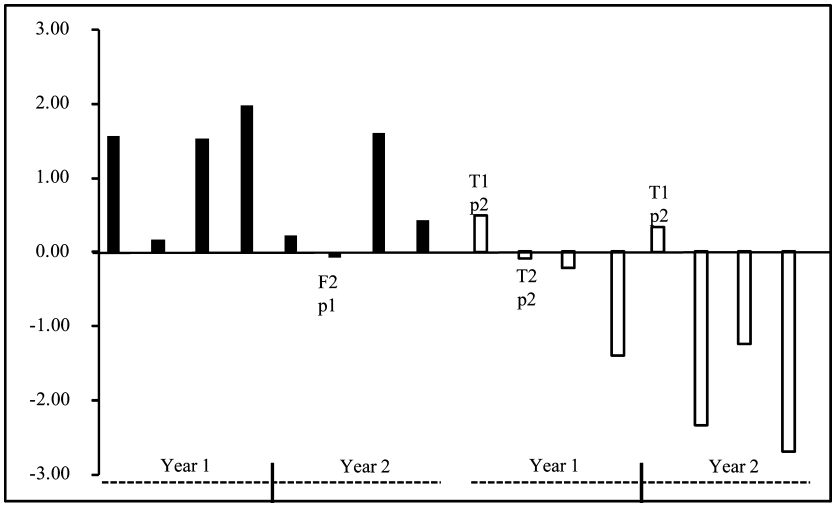

Figure 4. Ratio between the amount C_{29} and C_{27} in pasture samples, expressed as 3.6-C_{29}/C_{27}: (black bars) *F. nigrescens* type (F); (white bars) *T. alpinum* type (T). p1−p4 identify the experimental paddocks indicated in Figure 1.

cheese sample T3p2, collected in the second year of experimentation, which derived from cows grazing on paddock 2 that showed anomalous values of C_{29}/C_{27} at its first sampling (T1p2). No direct explanation can be found for the behavior of the F2p1 cheese sample in the first year of experimentation, but a general consideration should be made when such results are discussed. A plant is a highly nonhomogeneous matrix, due to the presence of different parts (stem, leaf, flower, and seed) that are in different proportions, depending on the phenologycal stage, and give a different contribution to the chemical composition. Moreover, the pasture is a very complex natural system, including several species. Pastures F and T were defined on the basis of the species providing the most contribution (*F. nigrescens* and *T. alpinum*) and dominating during the two years,[26] but their characteristics were certainly affected by the presence of other species that contributed, with their variability, to the results.

Fatty Acid Composition. Table 4 reports the results obtained from the 48 cheeses analyzed over the two years. The characteristics of the FA composition of both categories of samples were in agreement with the literature data of milk fat deriving from cows grazing in mountain pastures.[24,40] The mean values of F and T groups were in satisfactory agreement with those published by Falchero et al.,[25] who analyzed the same cheese samples of the first year of experimentation, but applied different extraction, methylation, and GC conditions. The highest values here obtained for the short-chain fatty acids in all of the groups of samples (e.g., C4 = 4.11, C6 = 1.95, C8 = 1.14, and C10 = 2.30, for F samples) with respect to those previously published (Table 5 in ref 25) (e.g., C4 = 2.69, C6 = 1.70, C8 = 1.02, and C10 = 2.10, for F samples) were due to the application of the gas chromatographic correction factors, which took into account the low response of the flame ionization detector to the molecules with a low number of carbon atoms.[41] The other differences were due to the highest separation ability of the GC column applied in this research that allowed a higher number of molecules to be detected and quantified, particularly branched (iso and anteiso), PUFA, and *trans* isomers. The data deriving from two years of experimentation (Table 4) showed that, among the fatty acids reported also by Falchero et al.,[25] the influence of pasture was statistically significant on a smaller number of fatty acids (C13, C15, C17, C17:1, VA, C18:1 *cis* 9, and C18:3). This behavior was probably due to the increased variability deriving from the presence of the results obtained during the second year of experimentation.

Table 4. Mean Values (Grams per 100 g FAME) and Standard Deviations of Fatty Acids of Cheese Samples Produced from Milk Deriving from the Two Vegetation Types (*F. nigrescens*, F; *T. alpinum*, T) in Two Different Years[a]

	vegetation type			year		
	F	T		1	2	
4	4.11 ± 0.44	3.87 ± 0.79		4.38 ± 0.51	3.60 ± 0.53	**
6	1.95 ± 0.21	1.85 ± 0.32		2.05 ± 0.23	1.75 ± 0.24	**
8	1.14 ± 0.13	1.08 ± 0.19		1.18 ± 0.15	1.04 ± 0.15	**
10	2.30 ± 0.29	2.18 ± 0.38		2.33 ± 0.34	2.15 ± 0.33	
10:1	0.28 ± 0.06	0.27 ± 0.05		0.30 ± 0.05	0.24 ± 0.04	**
12	2.66 ± 0.33	2.55 ± 0.44		2.76 ± 0.38	2.44 ± 0.33	**
12:1	0.05 ± 0.01	0.04 ± 0.01		0.05 ± 0.01	0.04 ± 0.01	**
13 iso	0.07 ± 0.02	0.07 ± 0.02		0.08 ± 0.01	0.06 ± 0.01	**
13	0.13 ± 0.03	0.16 ± 0.02	*	0.16 ± 0.02	0.13 ± 0.02	**
14 iso	0.16 ± 0.03	0.15 ± 0.03		0.17 ± 0.02	0.13 ± 0.01	**
14	9.72 ± 1.03	9.57 ± 1.01		10.14 ± 0.88	9.13 ± 0.90	**
14:1	0.80 ± 0.18	0.8 ± 0.15		0.93 ± 0.12	0.67 ± 0.08	**
15 iso	0.32 ± 0.06	0.32 ± 0.05		0.37 ± 0.03	0.27 ± 0.03	**
15 anteiso	0.64 ± 0.11	0.62 ± 0.09		0.70 ± 0.07	0.55 ± 0.05	**
15	1.18 ± 0.33	1.50 ± 0.30	**	1.49 ± 0.33	1.19 ± 0.30	**
16 iso	0.31 ± 0.04	0.29 ± 0.04	**	0.33 ± 0.02	0.27 ± 0.02	**
16	26.8 ± 1.71	26.99 ± 1.79		25.73 ± 1.24	28.00 ± 1.31	**
cis (7 + 9) 16:1	1.65 ± 0.14	1.69 ± 0.14		1.68 ± 0.18	1.67 ± 0.08	
17 iso	0.56 ± 0.05	0.55 ± 0.05		0.60 ± 0.03	0.51 ± 0.02	**
17 anteiso	0.47 ± 0.05	0.45 ± 0.04	*	0.49 ± 0.04	0.42 ± 0.02	**
17	0.76 ± 0.16	0.96 ± 0.14	**	0.93 ± 0.15	0.78 ± 0.17	**
17:1	0.29 ± 0.06	0.38 ± 0.06	**	0.37 ± 0.08	0.30 ± 0.06	**
18	9.84 ± 1.10	9.46 ± 0.90		9.25 ± 0.73	10.03 ± 1.11	**
trans (6 + 7 + 8) 18:1	0.27 ± 0.03	0.26 ± 0.05		0.25 ± 0.04	0.29 ± 0.03	**
trans-9 18:1	0.20 ± 0.01	0.19 ± 0.02		0.20 ± 0.02	0.19 ± 0.02	
trans-10 18:1	0.29 ± 0.03	0.30 ± 0.05		0.30 ± 0.05	0.29 ± 0.03	
trans-11 18:1	3.51 ± 0.62	3.23 ± 0.67	*	2.88 ± 0.51	3.85 ± 0.33	**
trans-12 18:1	0.27 ± 0.03	0.26 ± 0.03		0.27 ± 0.03	0.26 ± 0.02	
trans-16 18:1	0.31 ± 0.03	0.29 ± 0.04	*	0.28 ± 0.04	0.32 ± 0.03	**
cis (9 + 10) +*trans* (13 + 14 + 15) 18:1	22.05 ± 2.13	23.27 ± 1.95	*	22.90 ± 2.03	22.37 ± 2.21	
cis-11 18:1	0.62 ± 0.07	0.63 ± 0.06		0.64 ± 0.07	0.61 ± 0.05	
cis-12 18:1	0.10 ± 0.02	0.10 ± 0.02		0.12 ± 0.01	0.08 ± 0.01	**
cis-13 18:1	0.08 ± 0.01	0.08 ± 0.01		0.08 ± 0.01	0.08 ± 0.01	
cis-15 18:1	0.20 ± 0.02	0.19 ± 0.03		0.19 ± 0.03	0.20 ± 0.02	
Σ *cis*/*trans*+*trans*/*cis*18:2[b]	0.62 ± 0.06	0.59 ± 0.06	*	0.64 ± 0.06	0.57 ± 0.05	**
trans-11,*cis*-15 18:2	0.55 ± 0.07	0.49 ± 0.09	**	0.48 ± 0.08	0.56 ± 0.08	**
cis-9,*cis*-12 18:2 (LA)	1.57 ± 0.19	1.48 ± 0.13		1.48 ± 0.16	1.57 ± 0.16	*
20	0.17 ± 0.05	0.18 ± 0.04		0.21 ± 0.04	0.14 ± 0.02	**
18:3n-6	0.02 ± 0.004	0.02 ± 0.003	*	0.02 ± 0.004	0.02 ± 0.003	**
cis-9,*cis*-12, *cis*-15 18:3 (ALA)	1.24 ± 0.14	0.92 ± 0.16	**	1.03 ± 0.21	1.13 ± 0.22	*
cis-9,*trans*-11 18:2 (CLA)[c]	1.75 ± 0.19	1.71 ± 0.28		1.62 ± 0.24	1.84 ± 0.18	**
trans-11,*cis*-13 18:2 (CLA)[d]	0.13 ± 0.02	0.12 ± 0.02		0.12 ± 0.02	0.13 ± 0.02	**
trans/*trans* CLA isomers	0.04 ± 0.006	0.04 ± 0.010		0.04 ± 0.010	0.04 ± 0.006	**
20:2n-6	0.03 ± 0.011	0.04 ± 0.010		0.04 ± 0.009	0.03 ± 0.004	**
22	0.08 ± 0.034	0.08 ± 0.025		0.10 ± 0.023	0.06 ± 0.011	**
20:3n-6	0.05 ± 0.007	0.04 ± 0.009		0.05 ± 0.007	0.04 ± 0.004	**
20:4n-6 (ARA)	0.09 ± 0.011	0.08 ± 0.013	**	0.09 ± 0.013	0.08 ± 0.008	**
20:4n-3	0.05 ± 0.011	0.04 ± 0.011	**	0.05 ± 0.009	0.03 ± 0.010	**
20:5n-3 (EPA)	0.06 ± 0.013	0.05 ± 0.013	**	0.06 ± 0.010	0.04 ± 0.010	**
24	0.04 ± 0.015	0.05 ± 0.017		0.06 ± 0.015	0.04 ± 0.010	**
22:4n-3	0.02 ± 0.005	0.01 ± 0.006	*	0.01 ± 0.004	0.02 ± 0.007	**
22:5n-3 (DPA)	0.08 ± 0.017	0.07 ± 0.019	*	0.09 ± 0.015	0.06 ± 0.010	**

[a]Significant difference: *, $p < 0.05$; **, $p < 0.01$. [b]Sum of the peaks eluting in the region of the *cis*/*trans* and *trans*/*cis* isomers of C18:2 and C18:1-*cis*-16. [c]This peak can include *trans*-7,*cis*-9 and *trans*-8,*cis*-10, accounting for about 3% of *cis*-9,*trans*-11 CLA, according to Collomb et al.[40] [d]This peak can include some *cis*/*cis* CLA isomers, according to Kramer et al.[43]

Linear odd-chain fatty acids are principally derived from bacteria in the rumen, but about 10% originate from both the diet and the de novo synthesis in the mammary gland.[42] The highest values of C15, C17, and C17:1 observed in cheese

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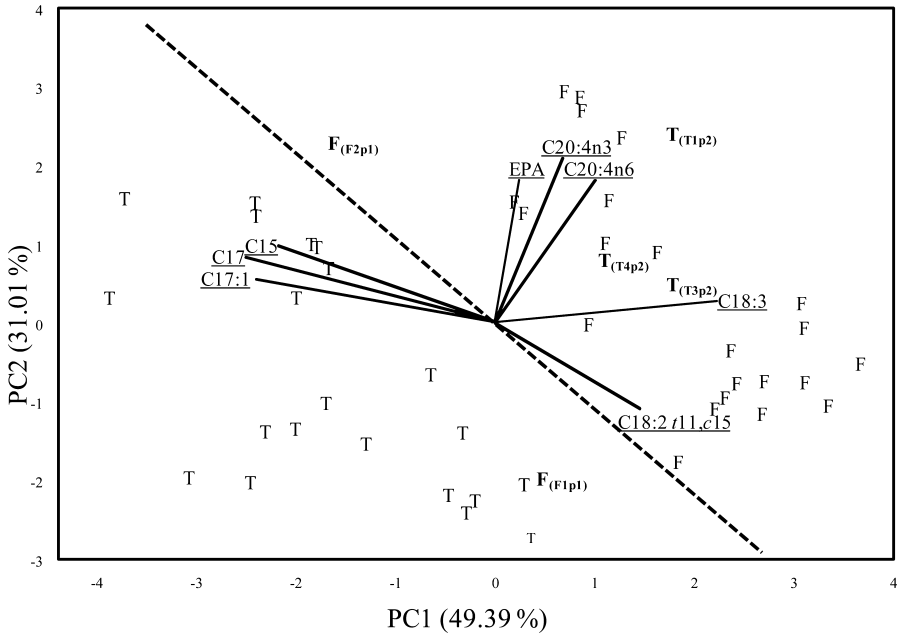

Figure 5. Biplot of the PCA of the 48 cheese samples and the variables providing differences statistically significant with $p < 0.01$ (*F. nigrescens*, F; *T. alpinum*, T). The dotted line separates the space of each category.

samples of category T were in accordance with the highest values of the same fatty acids previously detected in T pasture samples.[25]

The GC operative conditions applied allowed the separation of interesting isomers of both linoleic and conjugated linoleic acid. The tentative identification of these compounds was made according to the data published by different authors who used the same type of GC column and, for CLA isomers, compared the GC results with those obtained by argentation chromatography.[29,43−45] The high contribution of *trans*-11,*cis*-13 18:2 to the CLA isomers eluting within the retention times of *cis*-9,*trans*-11 C18:2 and *trans*,*trans* CLA was supported by the results obtained by Collomb[40] on milk samples deriving from cows grazing in highlands of the Swiss Alps, approximately at the same altitude as our experimentation. The contribution of *trans*-11,*cis*-13 18:2 to the total CLA content was about 6.5% (6.6 ± 0.8 in F samples and 6.5 ± 0.7 in T samples) and this result was in accordance with Collomb et al.[40] and Kraft et al.,[46] who found similar values in mountain milk samples (5−8%).

At the same time, a high content of *trans*-11,*cis*-15 C18:2, the highest linoleic acid isomer found in mountain milk samples accordingly to Collomb et al.,[21,24,40] was detected in all samples, supporting the hypothesis that this isomer was an intermediate product in the biosynthesis of *trans*-11,*cis*-13 18:2 from α-linoleic acid.

Among the other fatty acids detected, statistically significant differences were observed for C16 iso, C17 aiso, C18:1 *trans* 16, the *trans* isomers of C18:2, and the C20 and C22 PUFA, except for C20:3n-6. Docosahexaenoic acid was detected only in trace, in a small number of samples.

Principal component analysis (PCA) was applied to the two groups of cheeses using the FAs that showed differences statistically significant at $p < 0.01$ with respect to the pasture type (C15:0, C17:0, C17:1, *trans*-11,*cis*-15 C18:2, C18:3, C20:4n-6, C20:4n-3, and EPA). The aim of the application of this multivariate procedure was to highlight, on each sample, the effect of the information included in the most discriminating variables. As expected, good separation between T and F was obtained by taking into account the information contained in components 1 and 2, explaining 80% of the total variance (Figure 5). In addition, cheese samples F2p1, T1p2, T3p2, and T4p2, produced in the first year of experimentation, appeared quite far from the space of their own category and were the same samples recognized as the main outliers by the value of nonacosane/heptacosane ratio (Figure 3). This result strengthened that obtained by the hydrocarbon C_{29}/C_{27} ratio and demonstrated the real link existing between the pasture and the cheese characteristics.

The hydrocarbon and fatty acid composition of the cheese produced in this experimentation reflected the characteristics of the dairy products deriving from milk of cows grazing on highland pasture, without supplementation. Moreover, the particular experimental design made it possible to detect, in cheese samples, some constituents of both hydrocarbon and fatty acid fraction that were able to discriminate the origin of milk on the basis of two different pastures defined according to their dominating species: *F. nigrescens* and *T. alpinum*.

Because the whole data set included results of two different years of experimentation, it can be reasonably concluded that the compounds detected as biomarkers were satisfactorily reliable to withstand the possible sources of natural variation, that is, climatic conditions, characteristics of the herds, and botanical composition of the minor species of vegetation, that occurred during that period. Further studies, conducted over a longer time period, will be useful to confirm the results.

This research can be an example for a methodological approach applicable to the characterization of small dairy productions, typical of narrow geographical regions, but having a great importance for territory protection and economic development.

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